Variable effects of C-terminal tags on FLS2 function

Hurst, Charlotte; Turnbull, Dionne; Myles, Sally; Leslie , Kerry ; Keinath , Nana F. ; Hemsley, Piers

Published in:
Plant Physiology

DOI:
10.1104/pp.17.01700

Publication date:
2018

Variable Effects of C-Terminal Fusions on FLS2 Function: Not All Epitope Tags Are Created Equal[1][CC-BY]

Charlotte H. Hurst,[a,b] Dionne Turnbull,[a] Sally M. Myles,[a] Kerry Leslie,[a,b] Nana F. Keinath,[c,2] and Piers A. Hemsley[a,b,3]

[a]Division of Plant Sciences, School of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom
[b]Cell and Molecular Sciences, James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom
[c]Department of Plant Developmental Biology, Centre for Organismal Studies, Heidelberg University, 69120 Heidelberg, Germany

ORCID ID: 0000-0003-2950-0634 (P.A.H.)

Receptor-like kinases (RLKs) are the largest family of proteins in plants and are responsible for perceiving the vast majority of extracellular stimuli. Thus, RLKs function in diverse processes, including sensing pathogen attacks, regulating symbiotic interactions, transducing hormone and peptide signals, and monitoring cell wall status. However, despite their fundamental role in plant biology, very few antibodies are available against RLKs, which necessitates the use of epitope tags and fluorescent protein fusions in biochemical analyses such as immunoblot analysis and intracellular visualization. Epitope tags are widely used and are typically assumed to be benign, with no influence on protein function. FLAGELLIN SENSITIVE2 (FLS2) is the receptor for bacterial flagellin and often is used as a model for RLK function. Previous work implies that carboxyl-terminal epitope fusions to FLS2 maintain protein function. Here, a detailed complementation analysis of Arabidopsis (*Arabidopsis thaliana*) *fls2* mutant plants expressing various FLS2 C-terminal epitope fusions revealed highly variable and unpredictable FLS2-mediated signaling outputs. In addition, only one out of four FLS2 epitope fusions maintained the ability to inhibit plant growth in response to flg22 treatment comparable to that in the wild type or control untagged transgenic lines. These results raise concerns over the widespread use of RLK epitope tag fusions for functional studies. Many of the subtleties of FLS2 function, and by extension those of other RLKs, may have been overlooked or inappropriately interpreted through the use of RLK epitope tag fusions.

Receptor-like kinases (RLKs) form the largest gene family in plants (Shiu and Bleecker, 2001) and are the principal sensing mechanism for physical extracellular signals. Their roles in governing processes such as plant-microbe interactions (Macho and Zipfel, 2014), cell wall integrity (Voxeur and Höfte, 2016), hormonal status (Belkhadir et al., 2014; Xu et al., 2014), peptide signaling (Hara et al., 2007), and developmental processes (Clark et al., 1997; Fisher and Turner, 2007) are particularly well studied. Due to their core importance in plant biology, a very large body of work on RLKs has accrued, ranging from studies of their fundamental functions to transferal of RLKs to new species to provide novel pathogen resistance (Lacombe et al., 2010) or synthetic biology approaches to alter plant behavior (Brutus et al., 2010). Much of the work done to examine RLK function relies on biochemical approaches. In the absence of specific antibodies against the RLK of interest, protein sequences (epitopes) recognized by other antibodies must be fused to RLKs to enable detection. For this purpose, C-terminal fusion tags such as fluorescent proteins (Robatzek et al., 2006; Mbengue et al., 2016; Bücherl et al., 2017) and peptides, including HA (YPYDVPDYA; Dunning et al., 2007), MYC (EQKLISEEDL; Zipfel et al., 2004), or FLAG (DYKDDDDK; Sun et al., 2012), have proven popular. For the purposes of this report, any genetically encoded proteinaceous sequence added to a protein to enable detection by an antibody is referred to as an epitope tag. Epitope tags often are presumed to have no effect on function; however, a number of reports now suggest that N-terminal, C-terminal, or internal epitope placements can affect both protein subcellular localization and/or function. For instance, one in five mammalian proteins tagged with GFP do not colocalize with their native forms (Stadler et al., 2013). Moreover, any tag renders the potato (*Solanum tuberosum*) resistance protein R3a nonfunctional (Engelhardt et al., 2012), and GFP-tagged α-tubulin disrupts microtubule formation in Arabidopsis (*Arabidopsis thaliana*), leading to right-handed helical growth (Abe and Hashimoto, 2005). One report even details how epitope effects on

[1]This work was funded by UK Biotechnology and Biological Sciences Research Council grants BB/M024911/1 and BB/M010996/1 to P.A.H. and Deutsche Forschungsgemeinschaft grant KE 1719/2-1 to N.F.K.

[2]Current address: BioQuant, Im Neuenheimer Feld 267, Heidelberg University, D-69120 Heidelberg, Germany.

[3]Address correspondence to p.a.hemsley@dundee.ac.uk.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Piers A. Hemsley (p.a.hemsley@dundee.ac.uk).

P.A.H. and N.F.K. conceived the original research plan and designed the experiments; P.A.H., N.F.K., C.H.H., and D.T. supervised the experiments; C.H.H., S.M.M., D.T., N.F.K., K.L., and P.A.H. performed the experiments; P.A.H., C.H.H., S.M.M., D.T., and N.F.K. analyzed the data; P.A.H. and N.F.K. wrote the article with contributions from all coauthors.

www.plantphysiol.org/cgi/doi/10.1104/pp.17.01700

protein function vary depending on cell type, further complicating functional validation in one system being used to inform another (Jiang et al., 2012). Detailed work also indicates that multiples of an epitope tag, such as FLAG, MYC, or HA repeats, are more likely to disrupt function than a single tag (Georgieva et al., 2015).

FLAGELLIN SENSITIVE2 (FLS2) and BRASSINOSTEROID INSENSITIVE1 (BRI1), in conjunction with their coreceptor BRASSINOSTEROID-ASSOCIATED KINASE1 (BAK1), are the receptors for bacterial flagellin (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004; Chinchilla et al., 2007; Heese et al., 2007) and brassinosteroids (Friedrichsen et al., 2000; Li et al., 2002; Nam and Li, 2002), respectively. They are the most well-characterized leucine-rich repeat (LRR)-RLK pairings, with a range of tools and known signaling outputs available for their investigation. In particular, FLS2 and BAK1 are used frequently as the model system for LRR-RLK-mediated signaling during immune responses. FLS2 and BAK1 rapidly heterodimerize upon the perception of flagellin by FLS2 and downstream signaling responses are initiated, which include reactive oxygen species bursts, MAPK activation, and changes in gene expression (Chinchilla et al., 2007; Heese et al., 2007). Previous work has shown that BAK1, fused to various C-terminal epitopes, is able to interact with FLS2 in a flagellin-dependent manner but shows greatly reduced potency in terms of activating downstream signaling outputs (Ntoukakis et al., 2011). Interestingly, brassinosteroid signaling is largely unaffected by C-terminal tags on either BAK1 or BRI1 (Geldner et al., 2007; Ntoukakis et al., 2011), indicating that the effect of epitope tags on function is not easily predictable. Therefore, studies using BAK1 C-terminal fusions during the examination of pathogen-associated molecular pattern (PAMP)-triggered immune responses should be planned and interpreted appropriately (Ntoukakis et al., 2011). Historically, FLS2-epitope fusions have been assumed to be functional, as they confer flagellin responsiveness to the natural *fls2* mutant accession Wassilewskija-0 (Zipfel et al., 2004; Robatzek et al., 2006) and reportedly complement *fls2* mutant lines (Chinchilla et al., 2006). However, comparative assays of functionality or quantitative complementation analyses have not been shown explicitly, and, to the best of our knowledge, there are no published data on *fls2* loss-of-function mutant defects being restored to wild-type levels by physiologically relevant expression of FLS2 epitope fusions. We recently began working on FLS2 signaling, using our own and others' Arabidopsis lines expressing epitope-tagged FLS2 in *fls2* mutant backgrounds, and have discovered that C-terminally tagged FLS2 constructs do not behave in a consistent or predictable manner with respect to signaling outputs. Here, we demonstrate that three out of four FLS2 C-terminal epitope fusions are greatly impaired in their ability to restore flg22-mediated growth inhibition, suggesting that many FLS2 C-terminal epitope fusions are, at best, only partially functional. We present complementation assays for FLS2 C-terminal fusions as a resource for the community and identify the best constructs to use in future work.

RESULTS

The Effect of C-Terminal Epitope Tags on FLS2-Mediated Signaling Is Unpredictable

FLS2 recognizes flg22, the elicitor-active epitope of bacterial flagellin (Felix et al., 1999), and subsequently forms a dimer with BAK1 (Chinchilla et al., 2007; Heese et al., 2007). This interaction leads to increased activation of MAPK cascades, which is one of the earliest observable signaling outputs of PAMP-triggered immunity (PTI; Nühse et al., 2000). During work to characterize the flg22-induced responses of various forms of FLS2, we consistently observed that all lines expressing FLS2-mGFP6 fusions exhibited greatly reduced MAPK activation compared with that in wild-type Columbia-0 (Col-0) plants. Therefore, we set out to test whether C-terminal epitope tags impair FLS2 signaling or if these observations were a peculiarity of the mGFP6 tag. To this end, we used the previously published *fls2-101/FLS2*$_{pro}$*:FLS2-3xHA* line (Dunning et al., 2007) and two *fls2/FLS2*$_{pro}$*:FLS2-3xMYC-EGFP* lines (Mbengue et al., 2016). We also generated *fls2/FLS2*$_{pro}$*:FLS2-EGFP* lines as well as *fls2/FLS2*$_{pro}$*:FLS2* control lines without epitope tags. The constructs in these two lines, and the *FLS2*$_{pro}$*:FLS2-mGFP6* constructs described above, use the same promoter region and open reading frame described previously (Zipfel et al., 2004). For comparative purposes, linker, peptide epitope, and GFP sequences appended to the FLS2 C terminus used in this study are shown in Supplemental Figures S1 and S2. All generated epitope-tagged FLS2 transgenic lines were tested for *FLS2* expression, and those lines showing a range of mRNA expression to control for expression level effects were selected for further study. *fls2/FLS2*$_{pro}$*:FLS2* control lines were selected to cover the range of mRNA expression observed between the tagged lines under investigation and Col-0 to control for expression-level effects (Fig. 1; Supplemental Fig. S3). All plant lines displayed 3:1 antibiotic selection segregation in the T2, and quantitative PCR analysis indicated that each line carried the transgene integrated at a single locus. Comparison of transgene copy number in T3 lines with *FLS2* expression level showed very little correlation, suggesting that expression levels are likely dictated by transgene insertion site rather than transgene copy number (Fig. 1; Supplemental Fig. S3).

It was not possible to directly compare protein levels between the different tagged and untagged forms of FLS2, as the only α-FLS2 antibody available, raised against the extreme C terminus of FLS2 (Chinchilla et al., 2006; Hurst et al., 2017), shows variable and apparently reduced sensitivity toward epitope-tagged FLS2 (e.g. compare relative signal intensity for α-FLS2

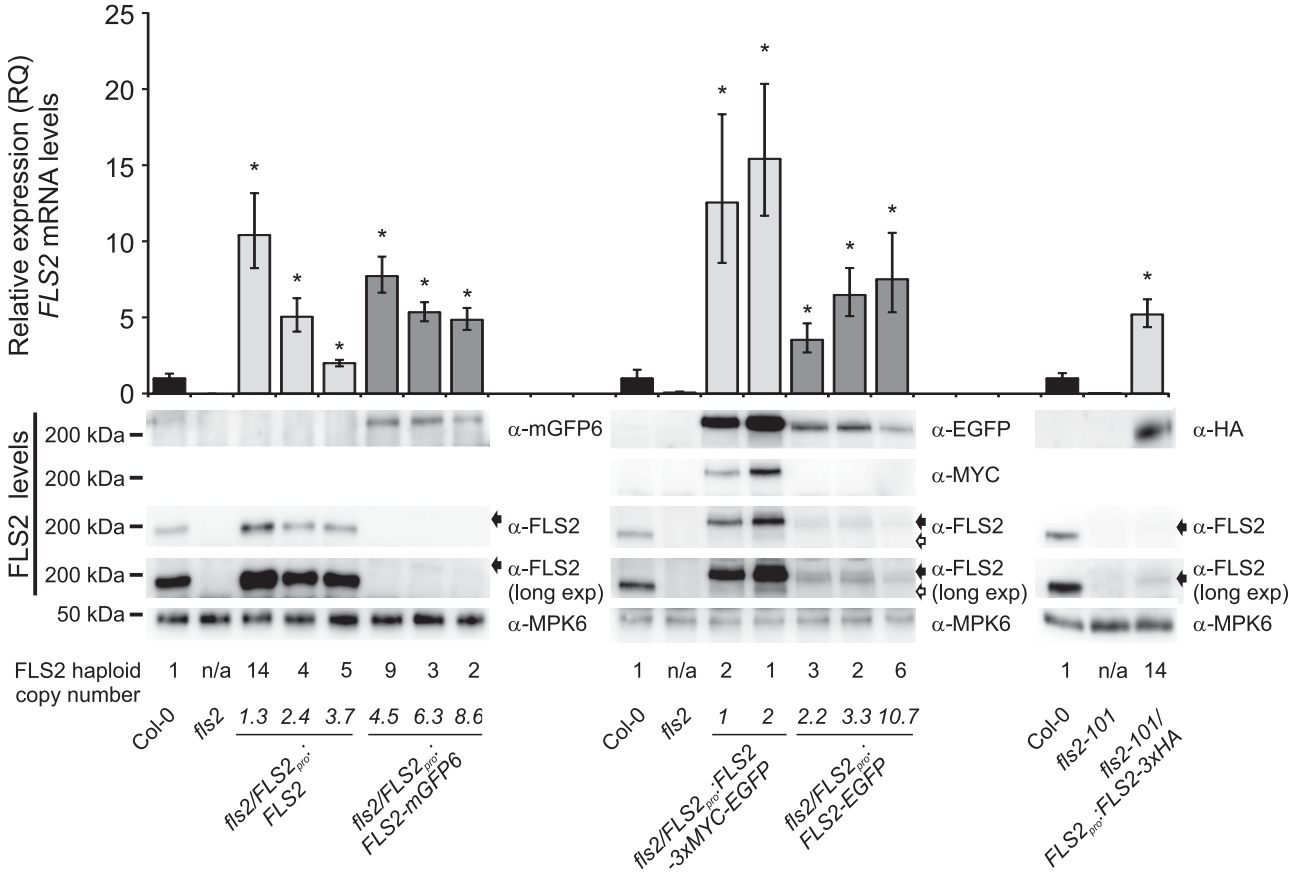


Figure 1. Expression of tagged and untagged forms of FLS2 in *fls2* mutant backgrounds. At top, the graph shows reverse transcription quantitative PCR analysis of *FLS2* expression levels after normalization to *PEX4* mRNA and relative to that in Col-0. Values were calculated using the $\Delta\Delta C_T$ method. Error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's *t* test. The graph shows data from one of two biological replicates, and asterisks indicate significant increases in *FLS2* mRNA levels compared with that in Col-0 in both biological replicates ($P < 0.05$). At bottom, immunoblot analysis is shown for levels of FLS2 (α-FLS2 antibody against the FLS2 C terminus) or tagged FLS2 (antibody directed against mGFP6, HA, EGFP, or MYC tag). MPK6 levels are shown as a loading control (α-MPK6). Long exp indicates long exposure for FLS2 (some signal will be saturated). Black arrows indicate FLS2 epitope fusion detected using α-FLS2 antibody, and white arrows indicate FLS2-sized bands likely originating from FLS2 epitope fusion cleavage.

and α-GFP in Fig. 1 and Supplemental Fig. S3 for *fls2/FLS2*$_{pro}$*:FLS2-3xMYC-EGFP* and *fls2/FLS2*$_{pro}$*:FLS2-EGFP*). This is likely due to the tags, by virtue of being attached to the C terminus of FLS2, partially disrupting the epitope recognized by the α-FLS2 antibody, with different tags affecting antibody binding to varying degrees. Therefore, it is not appropriate to draw quantitative comparisons between FLS2 levels in different lines using this antibody, which is important to note for future studies comparing different tagged and untagged FLS2 lines. A similar situation was observed for BAK1 when an antibody raised against the C terminus was used to probe tagged and untagged BAK1-expressing lines (Ntoukakis et al., 2011). Despite this, immunoblot analysis using antibodies against mGFP6, EGFP, MYC, or HA epitopes revealed signal at the appropriate M_r from the epitope-tagged FLS2-expressing lines, indicating that FLS2 protein was present. Relative *FLS2* mRNA and FLS2 protein levels within each set of lines correlated, with the exception of *fls2/FLS2*$_{pro}$*:FLS2-EGFP* #10.7, where high mRNA levels did not translate into higher protein levels. Although we do not have an explanation for this, sufficient FLS2-EGFP protein was produced to be detected by both anti-EGFP and anti-FLS2 immunoblot analysis. Interestingly, in FLS2-3xMYC-EGFP-expressing lines, an untagged FLS2-sized band was frequently observed, albeit weaker than the α-FLS2 signal from Col-0-derived FLS2 or full-length FLS2-3xMYC-EGFP, which is reactive with the FLS2 antibody but not MYC or GFP antibodies. This suggests the presence of a cleavage product where both 3xMYC and EGFP have been removed, leaving a form of FLS2 that closely resembles wild-type FLS2.

After flg22 treatment, FLS2-3xHA- and FLS2-mGFP6-expressing lines showed little MAPK6/3 activation, whereas MAPK6/3 activation in FLS2-EGFP-, FLS2-

3xMYC-EGFP-, and untagged FLS2-expressing lines were essentially indistinguishable from that in Col-0 (Fig. 2A). These data indicate that the presence of a C-terminal tag can have an impact on flg22-mediated FLS2 signaling outputs. RLKs have been shown to act in large multicomponent complexes (Jordá et al., 2016; Yeh et al., 2016), raising the possibility that the presence of the epitope tag in mGFP6- or HA-tagged lines could act as a general suppressor of PTI signaling. The elongation factor-Tu receptor EFR activates MAPK cascades in an almost identical manner to FLS2 during the perception of bacterial pathogens (Zipfel et al., 2006). Therefore, FLS2-3xHA- and FLS2-mGFP6-expressing lines were treated with the elongation factor-Tu-derived peptide elf18 and showed normal MAPK induction when compared with that in Col-0 (Fig. 2B). This demonstrates that the effect of mGFP6 or HA epitope tags on FLS2 is restricted to the outputs of FLS2-mediated signaling rather than PTI responses in general.

PAMP perception leads to transcriptional reprogramming and defense gene expression (Asai et al., 2002). In light of our conflicting MAPK activation data, we decided to test whether epitope-tagged FLS2-expressing lines exhibit changes in PAMP-induced gene expression. After flg22 treatment, typical early (*NHL10*; Zipfel et al., 2004) and late (*PR1*; Robatzek and Somssich, 2002) PAMP-induced genes were upregulated in Col-0 but not in *fls2* mutants (Fig. 2, C and D; Supplemental Fig. S4). However, *fls2* plants expressing epitope-tagged FLS2 variants showed variable PAMP-triggered gene induction when compared with that in Col-0 (Fig. 2, C and D; Supplemental Fig. S4). Lines expressing FLS2-3xHA or FLS2-mGFP6 showed little to no gene induction compared with that in Col-0, whereas lines expressing untagged FLS2 or 3xMYC-EGFP-tagged FLS2 appeared capable of activating all tested genes in response to flg22 (Fig. 2, C and D; Supplemental Fig. S4). FLS2-EGFP-expressing lines were capable of inducing gene expression but did not appear to be quite as effective as untagged or 3xMYC-EGFP-tagged lines at inducing early MAPK-mediated response genes such as *NHL10* (Fig. 2C; 1-h induction), whereas late salicylic acid-mediated response genes such as *PR1* were induced similar to that in Col-0 (Fig. 2D; Supplemental Fig. S4, C and D; 24-h induction). These data further substantiate the hypothesis that epitope tags can impact upon FLS2 functionality in terms of activating PAMP-responsive outputs, regardless of the mediating pathway, but not in a manner that can be readily predicted. Basal expression levels of *NHL10* and *PR1* are almost nil under non-flg22-challenged conditions; the presence of any *FLS2* transgene tested here, regardless of expression level or copy number, failed to elevate either *PR1* or *NHL10* expression compared with that in either unchallenged Col-0 or *fls2* mutant plants (Supplemental Fig. S5). This is similar to the observations made using MAPK activation and suggests that the transgenes do not affect plant physiology or outputs in the absence of flg22.

C-Terminal Epitope Tags Can Impair FLS2-Mediated Growth Inhibition

One of the responses to prolonged PAMP treatments such as flg22 is the inhibition of both seedling shoot and root growth, which often is used as an assay for the overall combined outputs and long-term effects of receptor pathway activation (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000). Therefore, in light of our unexpected data above on the varied functionality of FLS2 epitope fusions, we determined whether the lines tested also show variation in overall output resulting from long-term PAMP exposure. Interestingly, all FLS2 C-terminal fusions, with the exception of that in FLS2-3xMYC-EGFP-expressing lines, show impaired ability to inhibit seedling growth after flg22 treatment when compared with that in Col-0. Untagged FLS2-expressing lines essentially showed slightly greater or comparable growth inhibition to that in Col-0 (Fig. 3A), despite FLS2 being present at lower levels than in FLS2-3xMYC-EGFP-expressing lines. This implies that untagged FLS2 is still more active than the most potent tagged form of FLS2. These data indicate that the presence of a tag contributes more to the observed outcome of FLS2-mediated growth inhibition after flg22 treatment than FLS2 expression level (Fig. 3B).

Combined, these data indicate that C-terminal tagging of FLS2 can affect several PTI responses in a manner that cannot be easily predicted based solely on which epitope tag is used. Furthermore, the overall outcome of FLS2 signaling is consistently reduced in all FLS2 C-terminal fusion-expressing lines, with the potential exception of FLS2-3xMYC-EGFP-expressing plants. It is theoretically possible that the lines with mGFP6-, EGFP-, and HA-tagged FLS2, despite expressing *FLS2* and producing detectable FLS2 protein, do not produce stable FLS2 protein and that this leads to diminished responses compared with that in Col-0. Arguing against this is the strong MAPK activation and gene induction in the EGFP-tagged FLS2 lines and the partial rescue of growth inhibition in mGFP6- and EGFP-tagged FLS2 lines. This again suggests that the FLS2 antibody recognizing the C-terminal epitope, as used here and described previously (Chinchilla et al., 2006), is not suitable for comparing levels of the various tagged and untagged forms of FLS2. These data combined make the effect of a given tag on FLS2 outputs very difficult to predict.

DISCUSSION

Epitope fusions are a common tool in molecular biology and are indispensable for many cell biology applications or where antibodies are not available for a protein of interest. As the use of epitope tagging has become standard practice, it is easy to forget that the epitope itself may impact upon function by altering protein conformation, obscuring ligand-binding surfaces, or hindering protein-protein interactions. Deleterious

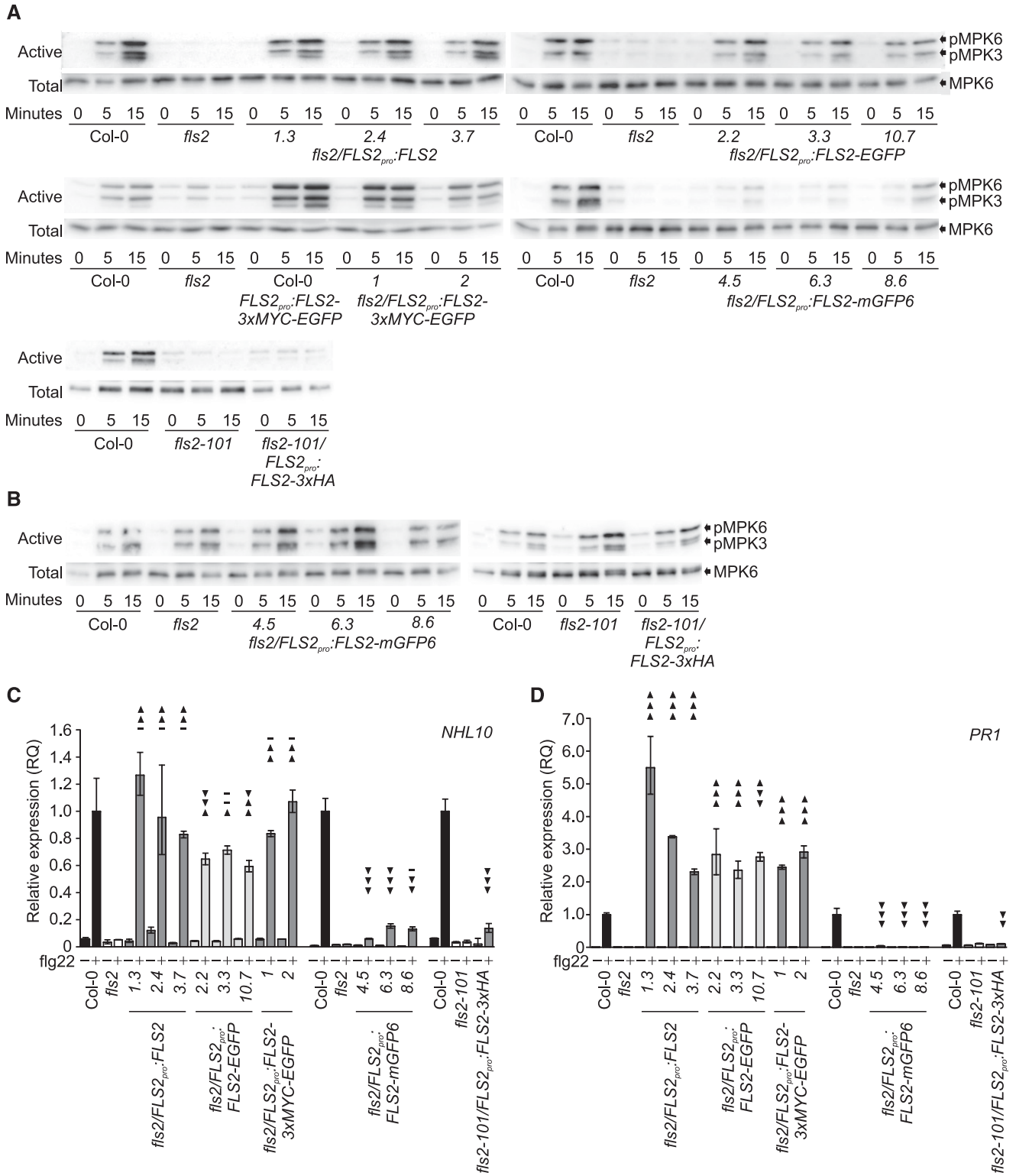


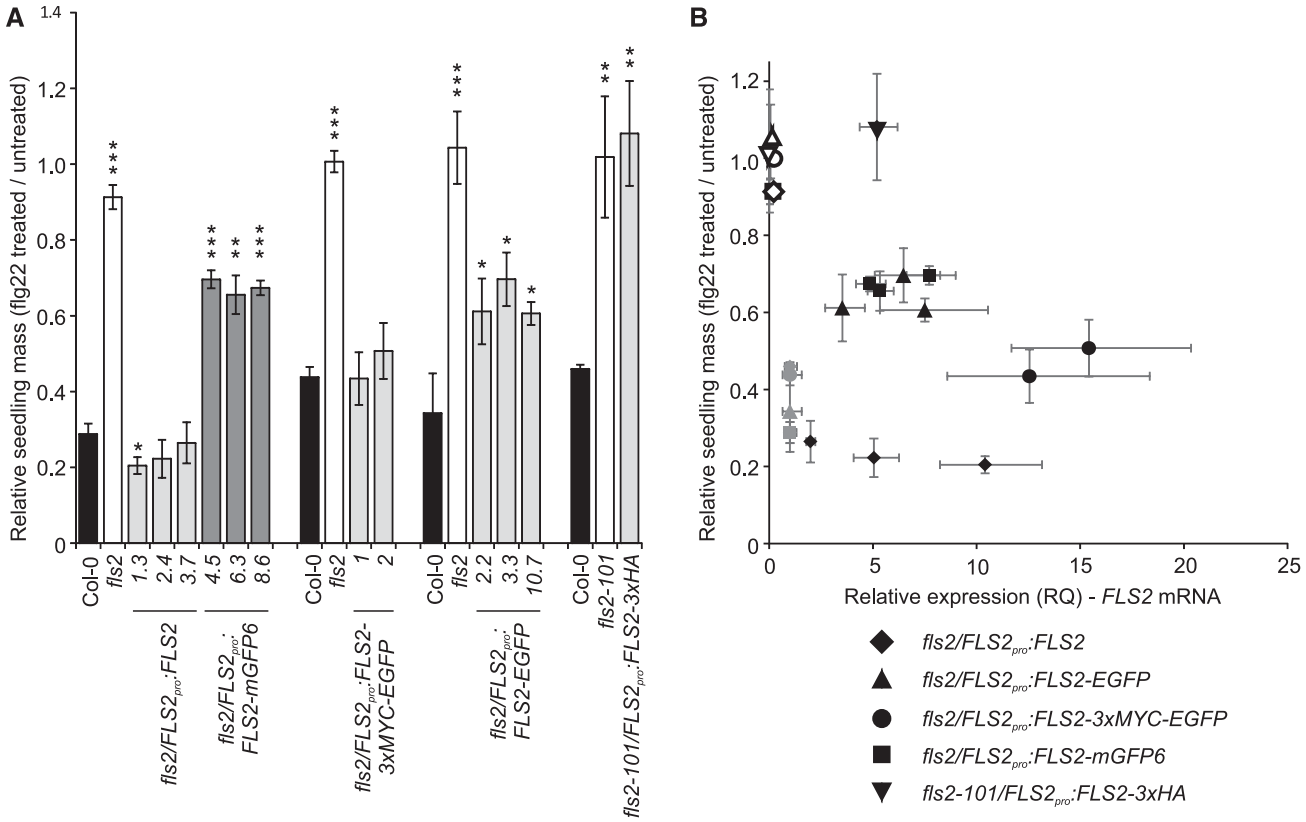
Figure 2. Plants expressing epitope-tagged FLS2 show variable MAPK activation and gene induction in response to elicitation by flg22. A, MAPK activation in *fls2* mutant seedlings expressing FLS2 tagged with 3xHA, mGFP6, EGFP, or 3xMYC-EGFP or untagged FLS2 in response to 100 nM flg22 as determined over time by immunoblot analysis. MAPK assays of Col-0 *FLS2*$_{pro}$*:FLS2-3xMYC-EGFP* lines were included for completeness but were not taken further, as no deleterious effects were observed and no conclusion about complementation could be drawn. B, MAPK activation in seedlings expressing 3xHA- or mGFP6-tagged FLS2 in response to 100 nM elf18, as determined over time by immunoblot analysis. Blots in A and B were probed with α-p42/44 (active MAPK; pMPK6/3) and α-MPK6 (total) as loading controls. C and D, Expression analysis of *NHL10* (C) and *PR1*

or anomalous effects of fused epitopes on protein function are not limited to FLS2, as reported here, but also have been identified for 20% of tested mammalian proteins (Stadler et al., 2013). The effects of fused epitope tags on RLK function have been shown in a study on BAK1 (Ntoukakis et al., 2011) and seem likely to also affect ERECTA (ER). An ER_{pro}:*ER-LUC* construct showed complementation in only 27% of recovered transformants compared with 100% complementation for an ER_{pro}:*ER* construct, but expression levels of ER-LUC fusions compared with wild-type ER levels were not determined and the significance or possible implications were not discussed further (Kosentka et al., 2017). Alongside BAK1 and BRI1, FLS2 is probably the best-studied RLK in plants, and much of the published work utilizes epitope-tagged forms. FLS2 has been reported to be functional when transformed into Wassilewskija-0 accession plants (a natural *fls2* mutant) as 3xMYC-EGFP or 3xMYC fusions (Zipfel et al., 2004; Robatzek et al., 2006), but, as those articles state, these data are gain-of-function analyses, not complementation assays; there is no reference for wild-type activity. In our work, we found that FLS2-3xHA- and FLS2-mGFP6-expressing lines were broadly impaired in all FLS2 responses tested. FLS2-EGFP-expressing lines showed an intermediate phenotype, while only those lines expressing FLS2-3xMYC-EGFP were able to complement the gene induction, MAPK activation, and seedling growth inhibition phenotypes of *fls2* mutants. Interestingly, FLS2-mGFP6- and FLS2-EGFP-expressing lines show similar growth inhibition despite FLS2-mGFP6 conferring minimal MAPK activation and reduced gene induction and FLS2-EGFP conferring wild-type levels of MAPK activation and only mildly affected gene induction. This suggests that, whereas MAPK and gene induction are differentially affected in these lines, other aspects of the flg22 response leading to growth inhibition not measured here are possibly less affected in FLS2-mGFP6-expressing lines.

Alternatively, absolute levels of MAPK activation and gene induction may not be closely correlated with the outcomes of growth inhibition experiments. This makes the effects of tags on each specific aspect or outcome of FLS2 function very difficult to predict. These unexpected and potentially variable effects of tags on FLS2 appear to have gone unreported, likely because the constructs are at least partially functional (Dunning et al., 2007; Hemsley et al., 2013; Figs. 1–3), unlike in the case of BAK1, where all tested C-terminal tags abolish function during PTI signaling (Ntoukakis et al., 2011). The effects of FLS2 expression levels on functional outcomes are reported to be nonlinear, with several orders of magnitude increase in expression required to increase measured outputs a few fold (Gómez-Gómez and Boller, 2000). In wild-type plants, FLS2 receptors have an EC_{50} of ~0.2 nM but a K_d of ~1.3 nM for flg22 (Bauer et al., 2001). This indicates that FLS2 signaling responses are saturated long before all receptor-binding sites are occupied; therefore, a 10-fold increase in FLS2 expression is not going to increase outputs 10-fold, particularly under treatment conditions commonly used, where flg22 concentrations range from 100 nM to 10 μM. In fact, our observations suggest that a 10-fold increase in FLS2 expression (e.g. $FLS2_{pro}$:*FLS2* line 1.3) has, at most, a 1.2-fold effect on growth inhibition and minimal effect on MAPK activation or gene expression compared with that in Col-0 or other $FLS2_{pro}$:*FLS2* lines with lesser untagged FLS2 expression. It is also a formal possibility that other factors, such as BAK1 or downstream components, are present in rate-limiting amounts. Therefore, elevated FLS2 expression would have limited additional effect, as extra activated FLS2 receptor would be reduced in its ability to transduce signal. As the *FLS2* promoter and coding sequence used in all of the lines studied here are identical and multiple independent transformants for each construct show the same phenotype regardless of FLS2 expression level, the observed variation must be the result of either the epitope tag or the vector T-DNA sequence. The vector effect likely can be excluded, as our own *fls2/*$FLS2_{pro}$:*FLS2-EGFP* and *fls2/*$FLS2_{pro}$:*FLS2* lines use the same vector series (Karimi et al., 2002).

Interestingly, a 3xHA tag (Dunning et al., 2007) of ~3 kD adopting a largely disordered conformation (Georgieva et al., 2015) is apparently more effective at reducing FLS2 responses compared with the ~27-kD, highly stable and structured mGFP6 or EGFP fusions. While this is outwardly counterintuitive, exposed, terminal regions of disorder, such as MYC or HA repeat peptide tags, have been suggested to have deleterious effects on protein function (Georgieva et al., 2015). BAK1-3xHA constructs also were unexpectedly impaired in BR responses despite being strongly expressed (Ntoukakis et al., 2011), indicating that C-terminal 3xHA tags may impair RLK function more than would otherwise be expected given their size. The linker between FLS2 and mGFP6 contains two Pro residues (Supplemental Fig. S2), and Pro is an amino acid known to produce inflexible linkers with reduced mobility (Radford et al., 1987). This may explain the difference in functionality observed between FLS2-mGFP6 and FLS2-EGFP, as the EGFP linker is predicted to be highly flexible and,

Figure 2. (*Continued*.)
(D). Expression levels were normalized to *PEX4* mRNA and relative to gene expression in Col-0 at 1 h (*NHL10*) or 24 h (*PR1*). Values were calculated using the $\Delta\Delta C_T$ method. Error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's *t* test. Data shown are representative of three independent biological replicates; significant increases (up triangles, $P < 0.05$), decreases (down triangles, $P < 0.05$), or no difference (–) in expression compared with that in Col-0 in each of the individual biological replicates are shown above each sample to illustrate consistency between biological replicates. The remaining two data sets are shown in Supplemental Figure S4.



C

Genotype	MAPK activation	NHL10 induction	PR1 induction	Growth inhibition
fls2/FLS2$_{pro}$:FLS2	o	++	++	o/+
fls2/FLS2$_{pro}$:FLS2-mGFP6	– –	– –	– –	–
fls2/FLS2$_{pro}$:FLS2-3xMYC-EGFP	o	++	++	o
fls2/FLS2$_{pro}$:FLS2-EGFP	o	o/–	++	–
fls2-101/FLS2$_{pro}$:FLS2-3xHA	– –	– –	– –	– –

Figure 3. flg22-mediated growth suppression can be impaired in plant lines expressing epitope-tagged FLS2. A, Average relative seedling mass (flg22 treated/untreated) of seedlings grown in 1 μM flg22 for 10 d (14 d post germination). Data are averages of at least two independent biological replicates. Error bars show SE. Asterisks denote statistically significant differences compared with that of the Col-0 control (*, $P < 0.1$; **, $P < 0.05$; and ***, $P < 0.01$) determined by one-way ANOVA and Tukey's HSD test. B, Growth inhibition data shown in A plotted against FLS2 mRNA expression data from Figure 1A. Gray and white data points represent Col-0 and *fls2/fls2-101* mutants, respectively, used as controls for each indicated genotype/experiment. *x* axis (*FLS2* expression) error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's *t* test; *y* axis (growth inhibition) error bars show SE. Statistically significant differences are as for A and Figure 1A but are omitted here for clarity. C, Summary table of FLS2 responses in all lines used in this study. Differences compared with that in Col-0 for each response are indicated: ++, strong increase; +, mild increase; o, no change; –, mild decrease; and – –, strong decrease.

therefore, less likely to sterically hinder downstream interactions (Supplemental Fig. S2). The most widely used and published FLS2 epitope fusion, demonstrated to be most active based on our data, is the FLS2-3xMYC-EGFP construct (Robatzek et al., 2006). In this construct, the highly disordered 3xMYC epitope (Georgieva et al., 2015) separates EGFP from FLS2 more than that in any other construct tested (Supplemental Fig. S2). This may reduce steric hindrance by GFP to a greater extent than that resulting from any

of the other constructs used here and allow for a more native conformation of the FLS2 C terminus and better access for interacting proteins. However, it is worth noting that, in FLS2-3xMYC-EGFP-expressing lines, an α-FLS2 reactive band is observed frequently at the size expected for full-length FLS2. This band is not detected by either α-MYC or α-GFP antibody, suggesting that this cleavage product contains a C terminus very similar to, or only slightly longer than, that of native FLS2. Therefore, it cannot be ruled out that this cleavage product may be able to function like untagged FLS2 and provide a greater degree of flg22 responsiveness in FLS2-3xMYC-EGFP-expressing lines than would be expected given the behavior of other epitope-tagged FLS2 lines. It should be noted that we have not tested this construct for complementation of every FLS2-mediated output described in the literature.

A possible explanation for the deleterious effects of epitope tagging on RLK outputs identified here or reported previously (Ntoukakis et al., 2011; Kosentka et al., 2017) involves a weak kinase activity (e.g. FLS2 or ER; Schwessinger et al., 2011) in the receptor/coreceptor pairing. It may well be the case that epitope tags impact upon all RLKs, but the effects are minor or unnoticed if both RLK partners are strong kinases (e.g. BRI1 or BAK1; Schwessinger et al., 2011) and able to compensate for the negative effects of the tag. Therefore, the use of RLK C-terminal fusions may reduce researchers' ability to properly observe and differentiate the effects of various mutant forms of RLKs on signaling or interactions. As a result, many intricacies and details of RLK signaling may have been obscured (Dunning et al., 2007; Sun et al., 2012; Hemsley et al., 2013; Kosentka et al., 2017), and potentially false-negative, uninterpretable, or inappropriate conclusions may have been reached in other studies that, therefore, have gone unpublished.

While this study indicates that untagged forms of FLS2 provide the best possible approach for testing the functionality of FLS2 variants, it is of course impossible to avoid epitope tags altogether. Much of the vital cell biology data on RLK localization and trafficking (Robatzek et al., 2006; Beck et al., 2012; Choi et al., 2013; Liang et al., 2013; Spallek et al., 2013; Smith et al., 2014; Mbengue et al., 2016) could not have been achieved without the presence of GFP or similar marker proteins, and the field would be much poorer for their absence. However, as FLS2 (this study), BAK1 (Ntoukakis et al., 2011), and ER (Kosentka et al., 2017) function have now all been shown to be affected by C-terminal epitope tags, we suggest that any functional study on RLKs using epitope tags should be designed and interpreted with care; the functionality of epitope tag fusions should not be assumed, and full quantitative complementation studies of each output of interest should precede further evaluation. Possible alternative solutions to C-terminal tagging not yet considered by the RLK field are the use of tags internal to the protein sequence or the use of innocuous tags (Georgieva et al., 2015). In the case of FLS2, the obvious and immediate solution is to base all future constructs on FLS2-3xMYC-EGFP (Robatzek et al., 2006), but it would be interesting to determine whether a 3xMYC or other long and flexible linker between the RLK of interest and GFP provides a generic solution to the issue of C-terminal RLK tags.

MATERIALS AND METHODS

Plant Lines and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) lines used are of the Col-0 ecotype. The *FLS2* mutant alleles, *fls2* (SAIL_691C4; Zipfel et al., 2004) and *fls2-101*, as well as *fls2-101/FLS2*$_{pro}$*:FLS2-3xHA* lines (Dunning et al., 2007) and *fls2/FLS2*$_{pro}$*:FLS2-3xMYC-EGFP* (Mbengue et al., 2016), have been described previously. *fls2/FLS2*$_{pro}$*:FLS2-mGFP6* (pMDC107 based; Curtis and Grossniklaus, 2003) and *fls2/FLS2*$_{pro}$*:FLS2-EGFP* (pH7FWG0 based; Karimi et al., 2002) were created for this study using a construct with the same promoter region and open reading frame of FLS2 lacking a stop codon, as found in the *FLS2*$_{pro}$*:FLS2-3xMYC-EGFP* construct and described previously (Zipfel et al., 2004; Robatzek et al., 2006). *fls2/FLS2*$_{pro}$*:FLS2* constructs were created using the same *FLS2* fragment as for mGFP6- and EGFP-tagged lines but with a stop codon introduced and cloned into pK7WG0 (Karimi et al., 2002). Transgenic Arabidopsis plants were generated by *Agrobacterium tumefaciens*-mediated floral dip transformation (Clough and Bent, 1998) and selected for homozygosity at T3. Plant material for the experiments was grown on 0.5× Murashige and Skoog (MS) medium and 0.8% (w/v) phytagar under a 16/8-h light/dark cycle at 20°C in MLR-350 growth chambers (Panasonic).

Transgene Copy Number Determination

Genomic DNA was extracted from Arabidopsis plate-grown seedlings (Edwards et al., 1991). Real-time PCR determination of copy number was performed using SYBR Green and the $\Delta\Delta C_T$ method as described previously (Bubner and Baldwin, 2004). Validated primer pairs used were against *NHL10* (endogenous control) and *FLS2* (target for copy number determination).

Gene Expression Analysis

Gene expression levels were analyzed using reverse transcription quantitative PCR. For this, 10 seedlings of each genotype 10 d post germination were treated with 1 μM flg22 for the indicated times. The 10 seedlings from each genotype at each time point for each treatment were pooled before further analysis. RNA was extracted using the RNeasy kit with on-column DNase digestion according to the manufacturer's instructions (Qiagen). Two micrograms of RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). All transcripts were amplified using validated gene-specific primers. Expression levels were normalized against *PEX4* (At5g25760; Wathugala et al., 2012). Each sample was analyzed in triplicate (technical replicates) for each primer pair within each biological replicate. Relative quantification (RQ) was achieved using the $\Delta\Delta C_T$ method (Yuan et al., 2006; Schmittgen and Livak, 2008). RQMIN and RQMAX define the range of possible RQ values calculated from the standard error of ΔCt using a 95% confidence interval derived from the t-distribution. Significant differences between samples in a biological replicate were defined by non-overlapping 95% confidence intervals. Fully independent biological replicates were performed over a period of 2 years, with each genotype only being present once in each replicate.

MAPK Activation

MAPK activation was performed essentially as described previously (Schwessinger et al., 2011). Six seedlings of each genotype 10 d post germination were treated with 100 nM flg22 or elf18 as appropriate for the indicated times in 2 mL of 0.5× MS medium. The six seedlings from each genotype at each time point for each treatment were pooled before further analysis. Fully independent biological replicates were performed over a period of 2 years, with each genotype only being present once in each replicate.

Immunoblot Analysis of Protein Levels

Proteins were extracted from pooled whole seedlings as described previously (Hurst et al., 2017) and blotted for active and total MAPK (Schwessinger et al., 2011) or FLS2 (Martínez-García et al., 1999; Hurst et al., 2017). Antibodies were supplied as follows: α-HA, Roche (11867423001); α-mGFP6, Santa Cruz Biotechnologies (sc-9996); α-EGFP, Roche (11814460001); α-MYC, Thermo (MA1-21316); α-p42/44, CST (#9101); and α-MPK6, Sigma-Aldrich (A7104).

Seedling Growth Inhibition

Seedling growth inhibition was performed essentially as described previously (Gómez-Gómez et al., 1999). Four days post germination, 10 seedlings with green cotyledons, erect hypocotyls, and emergent root of the named genotypes were transferred to 12-well plates (two seedlings per well), ensuring that the cotyledons were not submerged. Wells contained 2 mL of 0.5× MS liquid medium with or without 1 μM flg22. Seedlings were incubated for a further 10 d, and the fresh weight of pooled seedlings in each genotype for each treatment was measured and an average was calculated. flg22-treated and untreated weights for each genotype were calculated, and data are averages of these measurements for at least two biological replicates. Fully independent biological replicates were performed over a period of 18 months, with each genotype only being present once in each replicate.

Accession Numbers

Accession numbers are as follows: FLS2, At5g46330; BAK1, At4g33430; ER, At2g26330; NHL10, At2g35980; PR1, At2g14610; PEX4, At5g25760; MPK6, At2g43790; and MPK3, At3g45640.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Alignment of GFP sequences found in vectors used in this study.

Supplemental Figure S2. Alignment of FLS2 and appended C-terminal sequences in the constructs used in this study.

Supplemental Figure S3. Supporting data for Figure 1: biological replicates.

Supplemental Figure S4. Supporting data for Figure 2: biological replicates.

Supplemental Figure S5. Supporting data for Figure 2 and Supplemental Figure S4.

ACKNOWLEDGMENTS

We thank Andrew Bent for providing *fls2-101* and *fls2-101/FLS2$_{pro}$:FLS2-3xHA* and Col-0 control lines and Silke Robatzek for providing *fls2/FLS2$_{pro}$:FLS2-3xMYC-EGFP*. We thank Silke Robatzek, Georg Felix, and Cyril Zipfel for providing advice and helpful discussion during the preparation of the article.

Received November 27, 2017; accepted April 6, 2018; published April 23, 2018.

LITERATURE CITED

Abe T, Hashimoto T (2005) Altered microtubule dynamics by expression of modified alpha-tubulin protein causes right-handed helical growth in transgenic Arabidopsis plants. Plant J **43**: 191–204

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature **415**: 977–983

Bauer Z, Gómez-Gómez L, Boller T, Felix G (2001) Sensitivity of different ecotypes and mutants of Arabidopsis thaliana toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. J Biol Chem **276**: 45669–45676

Beck M, Zhou J, Faulkner C, MacLean D, Robatzek S (2012) Spatio-temporal cellular dynamics of the *Arabidopsis* flagellin receptor reveal activation status-dependent endosomal sorting. Plant Cell **24**: 4205–4219

Belkhadir Y, Yang L, Hetzel J, Dangl JL, Chory J (2014) The growth-defense pivot: crisis management in plants mediated by LRR-RK surface receptors. Trends Biochem Sci **39**: 447–456

Brutus A, Sicilia F, Macone A, Cervone F, De Lorenzo G (2010) A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. Proc Natl Acad Sci USA **107**: 9452–9457

Bubner B, Baldwin IT (2004) Use of real-time PCR for determining copy number and zygosity in transgenic plants. Plant Cell Rep **23**: 263–271

Bücherl CA, Jarsch IK, Schudoma C, Segonzac C, Mbengue M, Robatzek S, MacLean D, Ott T, Zipfel C (2017) Plant immune and growth receptors share common signalling components but localise to distinct plasma membrane nanodomains. eLife **6**: e25114

Chinchilla D, Bauer Z, Regenass M, Boller T, Felix G (2006) The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell **18**: 465–476

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JD, Felix G, Boller T (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature **448**: 497–500

Choi SW, Tamaki T, Ebine K, Uemura T, Ueda T, Nakano A (2013) RABA members act in distinct steps of subcellular trafficking of the FLAGELLIN SENSING2 receptor. Plant Cell **25**: 1174–1187

Clark SE, Williams RW, Meyerowitz EM (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell **89**: 575–585

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16**: 735–743

Curtis MD, Grossniklaus U (2003) A Gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133**: 462–469

Dunning FM, Sun W, Jansen KL, Helft L, Bent AF (2007) Identification and mutational analysis of *Arabidopsis* FLS2 leucine-rich repeat domain residues that contribute to flagellin perception. Plant Cell **19**: 3297–3313

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res **19**: 1349

Engelhardt S, Boevink PC, Armstrong MR, Ramos MB, Hein I, Birch PR (2012) Relocalization of late blight resistance protein R3a to endosomal compartments is associated with effector recognition and required for the immune response. Plant Cell **24**: 5142–5158

Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J **18**: 265–276

Fisher K, Turner S (2007) PXY, a receptor-like kinase essential for maintaining polarity during plant vascular-tissue development. Curr Biol **17**: 1061–1066

Friedrichsen DM, Joazeiro CA, Li J, Hunter T, Chory J (2000) Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. Plant Physiol **123**: 1247–1256

Geldner N, Hyman DL, Wang X, Schumacher K, Chory J (2007) Endosomal signaling of plant steroid receptor kinase BRI1. Genes Dev **21**: 1598–1602

Georgieva MV, Yahya G, Codó L, Ortiz R, Teixidó L, Claros J, Jara R, Jara M, Iborra A, Gelpí JL, (2015) Inntags: small self-structured epitopes for innocuous protein tagging. Nat Methods **12**: 955–958

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol Cell **5**: 1003–1011

Gómez-Gómez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J **18**: 277–284

Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T (2007) The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. Genes Dev **21**: 1720–1725

Heese A, Hann DR, Gimenez-Ibanez S, Jones AM, He K, Li J, Schroeder JI, Peck SC, Rathjen JP (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proc Natl Acad Sci USA **104**: 12217–12222

Hemsley PA, Weimar T, Lilley KS, Dupree P, Grierson CS (2013) A proteomic approach identifies many novel palmitoylated proteins in Arabidopsis. New Phytol **197**: 805–814

Hurst CH, Turnbull D, Plain F, Fuller W, Hemsley PA (2017) Maleimide scavenging enhances determination of protein S-palmitoylation state in acyl-exchange methods. Biotechniques **62**: 69–75

Jiang L, Teng GM, Chan EY, Au SW, Wise H, Lee SS, Cheung WT (2012) Impact of cell type and epitope tagging on heterologous expression of G protein-coupled receptor: a systematic study on angiotensin type II receptor. PLoS ONE **7**: e47016

Jordá L, Sopeña-Torres S, Escudero V, Nuñez-Corcuera B, Delgado-Cerezo M, Torii KU, Molina A (2016) ERECTA and BAK1 receptor like kinases interact to regulate immune responses in Arabidopsis. Front Plant Sci **7**: 897

Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci **7**: 193–195

Kosentka PZ, Zhang L, Simon YA, Satpathy B, Maradiaga R, Mitoubsi O, Shpak ED (2017) Identification of critical functional residues of receptor-like kinase ERECTA. J Exp Bot **68**: 1507–1518

Lacombe S, Rougon-Cardoso A, Sherwood E, Peeters N, Dahlbeck D, van Esse HP, Smoker M, Rallapalli G, Thomma BP, Staskawicz B, (2010) Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. Nat Biotechnol **28**: 365–369

Li J, Wen J, Lease KA, Doke JT, Tax FE, Walker JC (2002) BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell **110**: 213–222

Liang Y, Cao Y, Tanaka K, Thibivilliers S, Wan J, Choi J, Kang C, Qiu J, Stacey G (2013) Nonlegumes respond to rhizobial Nod factors by suppressing the innate immune response. Science **341**: 1384–1387

Macho AP, Zipfel C (2014) Plant PRRs and the activation of innate immune signaling. Mol Cell **54**: 263–272

Martínez-García JF, Monte E, Quail PH (1999) A simple, rapid and quantitative method for preparing Arabidopsis protein extracts for immunoblot analysis. Plant J **20**: 251–257

Mbengue M, Bourdais G, Gervasi F, Beck M, Zhou J, Spallek T, Bartels S, Boller T, Ueda T, Kuhn H, (2016) Clathrin-dependent endocytosis is required for immunity mediated by pattern recognition receptor kinases. Proc Natl Acad Sci USA **113**: 11034–11039

Nam KH, Li J (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell **110**: 203–212

Ntoukakis V, Schwessinger B, Segonzac C, Zipfel C (2011) Cautionary notes on the use of C-terminal BAK1 fusion proteins for functional studies. Plant Cell **23**: 3871–3878

Nühse TS, Peck SC, Hirt H, Boller T (2000) Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK 6. J Biol Chem **275**: 7521–7526

Radford SE, Laue ED, Perham RN, Miles JS, Guest JR (1987) Segmental structure and protein domains in the pyruvate dehydrogenase multienzyme complex of Escherichia coli: genetic reconstruction in vitro and 1H-n.m.r. spectroscopy. Biochem J **247**: 641–649

Robatzek S, Somssich IE (2002) Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev **16**: 1139–1149

Robatzek S, Chinchilla D, Boller T (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. Genes Dev **20**: 537–542

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc **3**: 1101–1108

Schwessinger B, Roux M, Kadota Y, Ntoukakis V, Sklenar J, Jones A, Zipfel C (2011) Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. PLoS Genet **7**: e1002046

Shiu SH, Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA **98**: 10763–10768

Smith JM, Salamango DJ, Leslie ME, Collins CA, Heese A (2014) Sensitivity to Flg22 is modulated by ligand-induced degradation and de novo synthesis of the endogenous flagellin-receptor FLAGELLIN-SENSING2. Plant Physiol **164**: 440–454

Spallek T, Beck M, Ben Khaled S, Salomon S, Bourdais G, Schellmann S, Robatzek S (2013) ESCRT-I mediates FLS2 endosomal sorting and plant immunity. PLoS Genet **9**: e1004035

Stadler C, Rexhepaj E, Singan VR, Murphy RF, Pepperkok R, Uhlén M, Simpson JC, Lundberg E (2013) Immunofluorescence and fluorescent-protein tagging show high correlation for protein localization in mammalian cells. Nat Methods **10**: 315–323

Sun W, Cao Y, Jansen Labby K, Bittel P, Boller T, Bent AF (2012) Probing the *Arabidopsis* flagellin receptor: FLS2-FLS2 association and the contributions of specific domains to signaling function. Plant Cell **24**: 1096–1113

Voxeur A, Höfte H (2016) Cell wall integrity signaling in plants: "To grow or not to grow that's the question." Glycobiology **26**: 950–960

Wathugala DL, Hemsley PA, Moffat CS, Cremelie P, Knight MR, Knight H (2012) The Mediator subunit SFR6/MED16 controls defence gene expression mediated by salicylic acid and jasmonate responsive pathways. New Phytol **195**: 217–230

Xu T, Dai N, Chen J, Nagawa S, Cao M, Li H, Zhou Z, Chen X, De Rycke R, Rakusová H, (2014) Cell surface ABP1-TMK auxin-sensing complex activates ROP GTPase signaling. Science **343**: 1025–1028

Yeh YH, Panzeri D, Kadota Y, Huang YC, Huang PY, Tao CN, Roux M, Chien HC, Chin TC, Chu PW, (2016) The *Arabidopsis* Malectin-Like/LRR-RLK IOS1 is critical for BAK1-dependent and BAK1-independent pattern-triggered immunity. Plant Cell **28**: 1701–1721

Yuan JS, Reed A, Chen F, Stewart CN Jr (2006) Statistical analysis of real-time PCR data. BMC Bioinformatics **7**: 85

Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428**: 764–767

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell **125**: 749–760